



## **New insights into the pathogenesis of Beckwith-Wiedemann and Silver-Russell syndromes: contribution of small copy number variations to 11p15 imprinting defects.**

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## **Contribution of Small Copy Number Variations to The Pathogenesis of Imprinted 11p15-Related Fetal Growth Disorders**

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Key words: genomic imprinting, copy number variation, 11p15 region, Silver-Russell syndrome, Beckwith-Wiedemann syndrome, fetal growth.

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## **Abstract**

The imprinted 11p15 region is organized in two domains, each of them under the control of its own imprinting control region (ICR1 for the *IGF2/H19* domain and ICR2 for the *KCNQ1OT1/CDKN1C* domain).

Disruption of 11p15 imprinting results in two fetal growth disorders with opposite phenotypes: the Beckwith-Wiedemann (BWS) and the Silver-Russell (SRS) syndromes. Various 11p15 genetic and epigenetic defects have been demonstrated in BWS and SRS. Among them, isolated DNA methylation defects (ICR1 gain of methylation or ICR2 loss of methylation in BWS and ICR1 loss of methylation in SRS) account for approximately 60% of patients.

To investigate whether cryptic Copy Number Variations (CNVs) involving only part of one of the two imprinted domains account for 11p15 isolated DNA methylation defects, we designed a Single Nucleotide Polymorphism array covering the whole 11p15 imprinted region and genotyped one hundred eighty five SRS or BWS cases with loss or gain of DNA methylation at either ICR1 or ICR2.

We describe herein novel small gain and loss CNVs in six BWS or SRS patients, including maternally-inherited *cis*-duplications involving only part of one of the domains in SRS and BWS patients. We also show that *i*) ICR2 deletions do not account for BWS with ICR2 loss of methylation and *ii*) uniparental isodisomy involving only one of the two imprinted domains is not a mechanism for SRS or BWS.

Those novel defects led new light in the regulation of 11p15 genomic imprinting.

## Introduction

Human chromosome 11p15.5 contains a cluster of imprinted genes that play a crucial role in the control of fetal growth<sup>1-4</sup>. This cluster is organized in two neighboring imprinted domains, the *IGF2/H19* and the *KCNQ1OT1/CDKN1C* domains, each of them under the control of its own imprinting control region, ICR1 and ICR2 respectively. Aberrant genomic imprinting of the 11p15 region has a pivotal role in both Beckwith-Wiedemann (BWS; MIM 130650) and Silver-Russell (SRS; MIM 180860) syndromes. BWS is characterized by pre-and/or postnatal overgrowth and other features including hemihyperplasia and an increased risk of childhood tumors. SRS is characterized by severe pre- and postnatal growth retardation, dysmorphic facial features, feeding difficulties and body asymmetry.

Various 11p15 molecular aberrations including genetic and epigenetic abnormalities have been demonstrated in BWS and SRS<sup>5-8</sup>. Among genetic defects, paternal isodisomy of the 11p15 region involving the two imprinted domains is a classical cause of BWS and accounts for 20-25% of BWS cases<sup>5, 9, 10</sup>, whereas maternal isodisomy has been reported in only one SRS case<sup>11</sup>. Approximately ten unbalanced translocations (gain CNV) involving both imprinted domains have also been described. When familial, these translocations result in a fetal growth retardation phenotype when maternally-inherited and an overgrowth phenotype when paternally-inherited, as reviewed in<sup>12</sup>. Nevertheless, epigenetic defects (i.e gain or loss of DNA methylation at either ICR1 or ICR2) are very frequent and account for approximately 60-70% of BWS and SRS patients. DNA methylation defects involving ICR2 (loss of methylation) result in BWS (60% of cases) whereas DNA methylation defects involving ICR1 result in both BWS (gain of methylation, 10% of cases) and SRS (loss of methylation, 50-60% of cases)<sup>5-7, 13</sup>. For a small percentage of those patients, the DNA methylation defect is secondary. Mutations/deletions in ICR1 *cis*-regulatory elements account for 20% of BWS patients with ICR1 gain of methylation<sup>14-18</sup>, whereas deletions in ICR2 *cis*-regulatory elements in BWS patients have been exceptionally reported<sup>19, 20</sup>. On the other hand, in a subset of BWS patients (25%) with

ICR2 loss of methylation and SRS patients (10%) with ICR1 loss of methylation, the loss of methylation involves imprinted loci other than 11p15 suggesting the dysregulation of an unidentified *trans*-acting factor controlling the establishment and/or the maintenance of genomic imprinting at several loci<sup>21-26</sup>. Altogether, these secondary DNA methylation defects (deletion of a *cis*-regulatory element or involvement of a *trans*-acting regulatory factor) account for a small group of BWS and SRS cases and the mechanism of the DNA methylation defect remains unexplained in most cases.

Conventional diagnostic tools used in 11p15 fetal growth disorders present either a low resolution level (> 500 kb), (*i.e* caryotype, FISH analysis) or high resolution level (< 5 kb), (sequencing, DNA methylation analysis, Multiplex ligation-dependent probe amplification, long-range PCR) and they might fail to recognize intermediate CNVs, ranging from 5 to 500 kb. Assessment of small segmental CNVs has not been systematically addressed in SRS and BWS patients with isolated DNA methylation defects. To investigate whether cryptic CNVs account for 11p15 isolated DNA methylation defects, we designed a customized Single Nucleotide Polymorphism (SNP) array (1536 SNPs) covering the whole 11p15 imprinted region. We genotyped a large series of one hundred eighty five SRS and BWS cases with loss or gain of DNA methylation at either ICR1 or ICR2. We describe herein novel gain and loss CNVs in both BWS and SRS patients.

## **Subjects and Methods**

### **Subjects**

This study was conducted in compliance with institutional guidelines for research studies in human genetics (approval no. 253/07, Alfred Hospital Ethics Committee and agreement numbers 681 and 682, Assistance Publique-Hôpitaux de Paris) and informed consent was obtained from participating individuals and/or their parents. The study population consisted of 50 ethnically matched control subjects and 185 patients with growth disorders caused by a DNA methylation defect of the 11p15 domain. A subset of BWS and SRS patients with abnormal DNA methylation at ICR1 was analyzed in a previous study <sup>18</sup>. Patients with cytogenetic abnormalities, 11p15 parental isodisomy or *CDKN1C* gene mutation and patients with a multilocus imprinting disorder <sup>21; 22</sup> were excluded from the study. Biparental inheritance of the 11p15 region was shown by Southern-blot and/or microsatellite analysis.

One hundred and thirteen patients were diagnosed with BWS. Seventy-eight of them displayed a loss of methylation at ICR2. Thirty-five BWS patients displayed a gain of methylation at ICR1. Nine BWS cases (six with ICR2 loss of methylation and three with ICR1 gain of methylation) were fetal cases (medically terminated pregnancies in 50% of cases and spontaneous abortion in 50% of cases). Five BWS cases were familial forms (one with ICR2 loss of methylation and four cases from two families with ICR1 gain of methylation).

Seventy-two patients were SRS patients who displayed loss of methylation at ICR1.

Parental DNA was available for 110 (59%) BWS or SRS patients. In 93 cases, DNA was available from both parents. Non-paternity was found in three BWS cases with ICR2 loss of DNA methylation and only maternal DNA was considered in those cases.

The clinical and molecular data of BWS and SRS patients are summarized in tables 1 and 2.

### **Methods**

#### **Selection of SNP probes and Oligo Pool Assay design**

An Oligo Pool Assay (OPA) of 1536 SNPs covering the whole 11p15

region (location on chromosome 11: position 1,970,084-2,869,522, NCBI 36/hg18 assembly) and including the two imprinted domains was designed. To allow a high success rate of genotyping, only SNPs with an Illumina SNP\_score >0.6 were selected among the 6214 polymorphisms present in dbSNP130 and located in the region of interest. SNPs already genotyped in the HapMap CEU population (our disease panel consists mostly of European individuals) were preferentially chosen. They represent 648 SNPs out of the 1536 final dataset. We selected SNPs dispersed homogenously along the domain with a final density of 1 to 3 probes per kilobase. Moreover, regions corresponding to the two imprinting control regions (ICR1 and ICR2) were enriched with a density of one probe every 300 bp. The customized OPA was used to genotype patients, their parents and some of the family members as well as control samples on the iScan System (<http://www.illumina.com>) according to the manufacturer's protocol.

#### **Preparation of SNP data**

We generated a total of ~ 735 000 genotypes in 473 samples. Genotypes were analyzed using the Illumina GenomeStudio software (<http://www.illumina.com>). After reclustering, 8 samples with a call rate <95% and 340 probes with excessive noise or aberrant clustering patterns (based on the GenTrain Score and manual inspection) were permanently excluded from further analysis. Following a series of quality control steps, an initial design using 1536 SNPs and 473 individuals was reduced to a final dataset of 1196 SNPs and 465 samples (including the 185 patients and 50 control subjects).

#### **CNVs detection by Illumina and QuantiSNP v2.3 softwares**

Analysis of CNVs was done based on log R ratio and B allele frequency values issued from the Illumina GenomeStudio and Beadstudio v3.1 softwares. Firstly, we used the cnvPartition v2.4.4 plug-in (<http://www.illumina.com>) implemented in the Illumina GenomeStudio software to identify CNVs. Given the density of probes in the OPA (one to three per kb), CNVs as small as 1 kb could be potentially detected. We performed a non-stringent analysis considering 3 consecutive probes, a minimum size of 1,000 bp and a threshold of 35 according to the

program. Because the cnvPartition algorithm uses an undocumented method of CNV detection, we also used the QuantiSNP v2.3 software developed by Colella *et al.* <sup>27</sup>. QuantiSNP based on Objective Bayes Hidden-Markov Model treats independently log R ratio and B allele frequency and has been shown to outperform other softwares <sup>28</sup>. The analysis was performed using default parameters <sup>27</sup> and a score (Log Bayes Factor) was assigned to each identified CNV; only CNVs with a stringent threshold score (Log Bayes Factor >30) have been considered. Secondly, to identify runs of homozygosity, we used the Homozygosity Detector plug-in (<http://www.illumina.com>) implemented in the Illumina BeadStudio v3.1 software as well as the QuantiSNP v2.3 software. The analysis was performed using the default parameters of the Homozygosity Detector program. All regions with more than 50 homozygous and a cutoff significance  $\chi^2 > 23.5$  were marked as runs of homozygosity. For the analysis using the QuantiSNP v2.3 software, only runs of homozygosity with a Log Bayes Factor > 30 were considered.

#### **Validation of identified CNVs by quantitative real-time PCR analysis and CGH array**

Quantitative real-time PCR: Validation of CNVs identified from the SNP array was performed by quantitative real time PCR. We designed at least 5 primer sets located within and outside the region of interest to confirm the CNVs and map their limits. Quantitative real time PCR was carried out by using a 7500 fast real time PCR system (ABI systems) and Fast SYBR green mix (Roche). Data expressed as the relative quantification were normalized to a region located in the neurogenin 1 gene in which no genetic variation has been described. Two normal individuals were included. All primers used in this study are listed in Supplementary table 1.

CGH array: Validation of some CNVs identified from the SNP array was performed using the 244K Agilent - Human CpG Island Microarray Kit (patient S72P) or the 4x180K Agilent-Human Genome Microarray Kit (patient H47P) (Agilent Technologies, Santa Clara, CA, USA).

#### **Bisulfite sequencing**



Bisulfite sequencing was performed as previously described <sup>18</sup>. Primers used in this study are listed in Supplementary table 1.

### **Karyotyping and FISH characterisation**

Chromosomes were prepared from peripheral blood lymphocyte cultures following standard procedures. Fluorescence in situ hybridization (FISH) using chromosome 11 specific subtelomeric probes (patient H47P) was performed according to the manufacturer's specifications (Abbott molecular/Vysis, USA). The BAC probes used in other FISH analyses include RP11-295K3 and RP3-416J11 (patient S72P) and CTD-2242D18 (patient L65P).

## Results

Several parameters were used to assess the likelihood of pathogenicity of a CNV in this study: *i*) the specific localization of a CNV within the 11p15 region (*i.e* the CNV involves key regulatory elements such as ICR2 in BWS patients with loss of methylation involving the centromeric domain or ICR1 in BWS and SRS patients with a DNA methylation defect of the telomeric domain). *ii*) the parental transmission of the CNV in familial cases: when inherited, a CNV inherited from a healthy parent was considered as potentially pathogenic. *iii*) copy-neutral CNVs (runs of homozygosity) were considered as potentially pathogenic as losses and gains, as they might reflect segmental uniparental isodisomy. *iv*) variants that are seen in healthy individuals are less likely to account for a patient's phenotype. To this purpose, the database of genomic variants (<http://projects.tcag.ca/variation/>) was consulted.

### ***New intermediate CNVs in BWS or SRS patients with DNA methylation defect at ICR1 or ICR2***

Six CNVs (2 deletions and 4 duplications) were identified in six patients, by using the cnvPartition v2.4.4 and/or the QuantiSNP v2.3 softwares (Table 3). Three CNVs (4 to 22 kb) were only identified with the cnvPartition software and they were not confirmed by quantitative PCR. Three CNVs, all duplications, were identified with both softwares and were confirmed by quantitative real-time PCR. Those three validated duplications were further characterized.

### **H47P BWS patient:**

The male patient was a first child, born after 38 weeks of gestation following ovarian stimulation. Prenatal sonography identified cystic hygroma and unilateral pyelectasis. Antenatal karyotype was normal. There was no familial history of BWS. At birth, the patient displayed severe macrosomia (birth length 56 cm, +3.4 SD; birth weight 5490 g, +5.9 SD), macroglossia, umbilical hernia and facial dysmorphism and the first months of post-natal life were marked by feeding difficulties. In early post-natal life, the patient was diagnosed with developmental delay, expressed primarily in the form of hypotonia and non-specific speech retardation. He walked at the age of 17 months. He underwent pyeloplasty for

ureteropyelic junction stenosis at the age of 1 year and glossoplasty at the age of 3 years. Physical examination at the age of 5<sup>1/12</sup> years showed a slight asymmetry of the face, lower limbs and kidneys and advanced growth (+3.5 SD for height and +4.5 SD for weight).

Analysis of the 11p15 region showed a partial gain of methylation at ICR1 [methylation index at 72% (normal: 53.9 ± 2%)] with a normal methylation status at ICR2 [methylation index at 45% (51.6 ± 2.5%)]. Analysis of SNP array data (Log R Ratio and B allele frequency) identified a duplication (Figure 1A). The duplication covered the whole *IGF2/H19* domain, involving the most telomeric probe (position 1,970,084, NCBI36/hg18 Assembly), with a centromeric breakpoint located within the 3' end of the *CD81* gene (between position 2,374,469 and 2,375,108). Quantitative PCR showed that the duplication extends to the telomeric end of the short arm (Figure 1B) refining the size of the duplication to approximately 2.4 Mb. SNP analysis showed that the duplication involved the paternal allele (Figure 1C). CGH array confirmed the telomeric duplication at 11p15.5 and also identified a 2 Mb telomeric deletion at 11q25 (breakpoint between position 132,431,292 and 134,432,465) (Figure 1D). Metaphase FISH analysis of H47P with a 11p subtelomeric probe showed signals at both telomeres of a derivative chromosome 11, whereas FISH analysis using a 11q subtelomeric probe confirmed a deletion of 11qter by showing a fluorescent signal on the normal chromosome 11 only (Figure 1E). FISH analysis in parents identified a pericentric inversion in the father, with the 11p subtelomeric probe hybridizing the end of 11q and the 11q subtelomeric probe hybridizing the end of 11p on the inverted chromosome (Figure 1E). As the size of the inverted segment is in the range of 130 Mb, the karyotypes were considered as normal in both H47P and his father (data not shown). This observation shows that partial trisomy 11p15 and partial monosomy 11q25 in H47P resulted from unequal crossing over during paternal meiosis.

### **S72P SRS patient:**

The female patient was the second child, conceived naturally from non consanguineous parents (Figure 2A). At birth (term of 36 weeks), she displayed intrauterine growth retardation [birth weight of 1690 g (-2.6 SD),

birth length of 39.5 cm (-4.4 SD)] with relative macrocephaly (-0.4 SD). Early postnatal life was marked by severe feeding difficulties and growth deceleration (-3 SD at the age by 7 months and -3.7 SD by the age of 15 months) with a BMI of -2 SD. SRS was diagnosed during the second year of life on the basis of postnatal growth retardation with conserved head circumference, bossed forehead, clinodactyly of the fifth digits, body asymmetry and feeding difficulties. The parents and the older brother were clinically normal and there was no familial history of SRS on the maternal side.

Analysis of the 11p15 region showed a partial loss of methylation at ICR1 (methylation index at 25%) with a normal methylation status at ICR2 (methylation index at 47%). Analysis of SNP array data identified a maternally-inherited duplication covering part of the *IGF2/H19* domain, including the *H19* gene and ICR1 but not the *IGF2* gene (Figure 2B). Characterization of the duplication by quantitative PCR and CGH array estimated its size between 562 and 575 kb with the telomeric breakpoint located between 1,522,259 and 1,530,602 bp (within the *HCCA2* gene) and the centromeric breakpoint located between 2,092,578 and 2,097,357 bp (Figures 2C and 2D). Two color FISH analysis of S72P and her mother with two BAC probes (RP11-295K3 within the duplication and RP3-416J11 outside the duplication) excluded an unbalanced translocation and showed an increased signal at 11p with RP11-295K3, providing indirect evidence for *cis*-duplication of the affected region (Figure 2E). FISH on S72P's interphased nuclei clearly showed three hybridization signals confirming the *cis*-duplication (Figure 2F). Methylation analysis in the mother showed a gain of methylation at ICR1 (methylation index at 68%). The mother's phenotype was normal and no clinical history in early childhood was indicative of BWS. Both maternal grandparents had normal phenotypes and there was no familial history suggesting SRS or BWS cases. Assuming that the grand paternal allele was methylated in the mother, we performed genomic and bisulfite sequencing of the ICR1 B6 repeat (1,980,297-1,980,616 bp) in S72P and her mother. An allelic imbalance was observed for the informative rs61383602 SNP in the mother, suggesting that the C allele was the duplicated allele (Figure 2 G). Bisulfite

sequencing showed that in the mother, the duplicated C allele peak was methylated confirming that the duplication originated from the maternal grandfather (Figure 2G).

**Familial L65P BWS case:** The family was previously reported as a clinical description in 1983 <sup>29</sup>. All the affected subjects were conceived from normal sisters (Figure 3A). Patient L65P (III.7, Figure 3A) was born after 40 weeks of a normal gestation from non consanguineous parents. The clinical presentation at birth included macroglossia, exomphalos, hypospadias, bilateral cryptorchidism, bilateral inguinal hernias, posterior helical pits and severe neonatal hypoglycemia. Patient L65P had no macrosomia at birth (birth weight 3200 gr, -0.6 SD) but then displayed advanced growth (+ 2.5 SD at the age of 5 years) with a final height at 196 cms (+ 2.8 SD). The familial history includes three other cases. The first pregnancy of individual II.1 was complicated at five months of gestation by acute hydramnios resulting in fetal death. Pathologic findings of the male fetus (III.1) included exomphalos, adrenal cytomegaly and leydigian hyperplasia. A second pregnancy was terminated after 25 weeks of gestation after ultrasound examination showed an exomphalos. The first pregnancy of individual II.4 was also terminated after 18 weeks of gestation after ultrasound diagnosis of exomphalos. Pathologic findings of the female fetus (III.8) included exomphalos, adrenal cytomegaly and ovarian hyperplasia. A second pregnancy resulted in spontaneous abortion at 9 weeks. Analysis of the 11p15 region showed a complete loss of methylation at ICR2 in patient III.7 whereas the ICR2 methylation status in the mother (II.3) and her two sisters (II.1 and II.4), obligate carriers, was normal (Figure 3B). The ICR2 methylation pattern was also normal in the individuals whom DNA was available, including the maternal grandmother (Figure 3B). Search for a deletion involving ICR2 was negative (PCR and quantitative PCR) and this hypothesis was unlikely as the ICR2 DNA methylation profile was normal in L65P's mother and her sisters. ICR2 was also sequenced (position 2,677,063 to 2,679,536 bp) and no small deletions or mutations were identified.

Analysis of SNP array and Log R Ratio data identified an approximately 50 kb duplication ranging from approximately 2,485,000 to 2,533,000 bp

(Figure 3C), involving the 3' part of intron 1, exon 2 and 5' part of intron 2 of the *KCNQ1* gene (isoform NM000218.2). The duplication was confirmed by quantitative PCR with the telomeric breakpoint located between 2,472,581 and 2,486,398 bp and the centromeric breakpoint located between 2,526,347 and 2,540,424 bp (Figure 3D). The duplication was also found in the three sisters (obligate carriers) and also in unaffected individuals II.5 and III.5. Subject I.1 did not display the duplication (Figures 3C and 3D), suggesting that the duplication was transmitted from L65P's maternal grandfather (DNA not available) to the five siblings (II.1 to II.5). The duplication was also absent in the only unaffected child (III.3) born from one (II.1) of the three sisters (Figures 3A and 3D). FISH analysis with the CTD-2242D18 probe (position: 2,469,461-2,575,865) did not detect an increased hybridization signal on chromosome 11. It is likely that the small size of the duplication prevented its identification by FISH. The absence of an extra signal on another chromosome is in favor of a 11p15 *cis*-duplication.

As the duplication encompasses two CpG islands, we investigated the methylation status of those CpG islands in three control subjects, L65P, his mother (II.3) and his maternal grandmother (I.1). Bisulfite sequencing analysis of the CpG island in position 2,510,680-2,510,907 showed that both parental alleles were unmethylated (Figure 3F) in control subjects, patient L65P and subjects II.3 and I.1. Analysis of the CpG island in position 2,511,982-2,512,194 showed that the CpG island was predominantly methylated with no allele-specific DNA methylation in controls (Figure 3F). In patient L65P, subjects II.3 and I.1, the DNA methylation pattern was the same as in control subjects.

### ***Small CNVs in BWS patients with gain of DNA methylation at ICR1***

A few ICR1 deletions have been previously reported, essentially in BWS patients. They are small in the range of 1-2 kb and as they involve very few markers, they might be missed by the CNV detection methods. We therefore performed an accurate analysis of respective imprinting control regions in patients with abnormal DNA methylation at ICR1 or ICR2, assuming that homozygous calls (B allele frequency) with a reduced copy

number (log R ratio) for SNPs mapping to ICR1 (1,976,000-1,981,000 bp) or ICR2 (2,676,000-2,679,000 bp) will reflect the occurrence of a hemizygous deletion.

Three BWS patients with ICR1 gain of methylation displayed a low log R ratio for two or three SNPs located within ICR1 (Figure 4A). Two of them, H32P (previously reported, <sup>18</sup>) and H50P presented as apparently sporadic BWS cases. H63P had a familial history of Wilms tumors on the maternal side (Figure 4B). H63P's mother herself displayed a phenotype indicative of BWS, including macrosomia at birth, organomegaly and body asymmetry. She also developed a Wilms tumor when she was 5 years old. Deletions were characterized by long range PCR, quantitative PCR and sequencing (Figures 4C, 4D and 4E). All deletions were maternally-inherited (Figure 4C). The two 1834 bp deletions (H32P and H50P) fusion blocks B6 with B3, removing two CTCF binding sites but involve different breakpoints (Figure 4F and Table 4). The 2240 bp H63P deletion fusions blocks B6 with B2 and removes CTCF binding sites 2, 3 and 4 (Figures 4E, 4F and Table 4).

No ICR1 deletion was detected in a series of 72 SRS patients displaying ICR1 loss of methylation. Similarly, investigation of a large series of 78 BWS with ICR2 loss of methylation did not identify any deletion.

### ***Copy neutral CNVs***

Copy neutral CNVs correspond to runs of homozygosity without any change in copy number. They might reflect uniparental isodisomy (UPiD) or autozygosity. UPiDs previously identified in BWS and SRS patients involve both ICR1 and ICR2 and display a mosaic pattern. The density of the SNP array and the availability of parental DNA for 60% of patients made possible the identification of segmental UPiDs. Analysis of the data for shift in B allele frequency for heterozygous SNPs (not centered at 0.5), associated with a normal log R ratio and mendelian inconsistency (when parental DNA was available) did not identify mosaic segmental UPiD in SRS or BWS patients with loss or gain of DNA methylation at either ICR1 or ICR2.

However, runs of homozygosity, ranging from 86 to 335 kb (156 to 592

probes) were identified in the various groups of patients and controls (Figure 5). Although runs of homozygosity were identified within the whole 11p15 region, two locations were overrepresented after analysis with the two softwares. The first region (position 2,170,000 to 2,270,000 bp), encompassing the paternally-imprinted *ASCL2* gene, was homozygous in 17% of both patients and controls (Box A in Figure 5 and supplementary Table 2). The second region (position 2,270,000 to 2,370,000 bp), encompassing the *C11ORF21* and *TSPAN32* genes, was homozygous in 23% and 15% of patients and controls respectively (Box B in Figure 5 and supplementary Table 2).



## Discussion

Recent modifications to SNP arrays enhance the opportunity to discover CNVs along with concomitant SNP genotypes. We designed a custom SNP array to target the analysis to the 11p15 region including the two imprinting control regions and investigate whether intermediate lesions (which cannot be recognized by routine diagnostic tools) account for a subset of 11p15 growth disorders with abnormal DNA methylation at either ICR1 or ICR2. A SNP array was favored to CGH array as it has the ability to (i) simultaneously measure both intensity differences and allelic ratios and therefore to investigate both DNA copy-number and copy-neutral variation and (ii) identify the parental origin of CNVs.

- We identified three novel duplications which have not been previously described.

Two duplications involved the *IGF2/H19* domain. In the H47P BWS case, CGH showed that a 2 Mb deletion of the distal part of 11q was associated with an apparently *de novo* 2.4 Mb duplication of the whole *IGF2/H19* domain on the paternal chromosome. FISH analysis of H47P and his parents demonstrated that H47P displayed recombination of a paternally-inherited pericentric inversion of chromosome 11, with a large inverted segment of approximately 130 Mb (i.e more than 95% of the chromosome). A few 11p pericentric inversions have been previously described<sup>30-32</sup>, some of them with a predominant BWS phenotype<sup>30</sup>. Those previously described pericentric inversions led to the duplication of the whole 11p15 imprinted domain with breakpoints between 11p13 and 11p15.1. The H47P case is unique as the duplication of the short arm involves only part of the 11p15 imprinted region (i.e the *IGF2/H19* domain). The deletion of 11q25 involves 10 genes and might account for the developmental delay.

The second duplication of the *IGF2/H19* domain represents the first observation of a genetic abnormality within the *IGF2/H19* domain in SRS. The S72P *cis*-duplication involves only part of the *IGF2/H19* domain (the imprinting control region and the *H19* gene) and results in a SRS phenotype only if maternally-inherited when there is no phenotype upon paternal transmission. Both the parental transmission pattern and the

phenotype are different from previously reported ICR1 duplications<sup>12, 33, 34</sup>. *Cis*-duplications (0.3 to 1.8 Mb in size) involving the whole *IGF2/H19* domain have been previously reported in two familial<sup>12, 34</sup> and one sporadic<sup>33</sup> BWS cases. They resulted in a BWS only if the paternal chromosome was involved. The main difference between the S72P duplication and other duplications of the *IGF2/H19* domain (<sup>12, 33, 34</sup> and the H47P case) is that the S72P duplication involves ICR1 and the *H19* gene but does not involve the *IGF2* gene. Hence, a partial maternal *cis*-duplication of the *IGF2/H19* domain results in a SRS phenotype (S72P case) whereas a maternal *cis*-duplication involving the whole *IGF2/H19* domain does not result in any phenotype<sup>12</sup> (Supplementary figure 1). Although two copies of the active maternal *H19* gene are expressed in both cases, the chromatin organization is likely to be different. In the partial *cis*-duplication, one maternal *H19* gene is not engaged in a *cis*-effect. It was recently shown that, apart from a *cis*-effect, the *H19* non-coding RNA also regulates the expression of *Igf2* by a *transacting* mechanism<sup>35</sup>. Indeed, *H19* transgenic expression is able to rescue the overgrowth phenotype of mice with targeted deletions of the *H19* gene and also to reduce *Igf2* expression<sup>35</sup>. We hypothesize that an extra copy of a functional maternal *H19* gene (not engaged in a *cis*-effect) can affect the expression of *IGF2* by a *transacting* mechanism and result in growth retardation. Two deletions within or overlapping the S72P duplicated region have been registered as rare variations, in apparently normal subjects (<http://projects.tcag.ca/variation/>; variations 29891, 29893), but the parental origin of these deletions are not known.

The third duplication case is a familial BWS case with ICR2 loss of methylation, involving four affected subjects conceived from three phenotypically normal sisters. The pattern of transmission of this *cis*-duplication supports its pathogenicity with no phenotype when the duplication is transmitted through the male germline and a BWS phenotype when the duplication is transmitted through the female germline. Moreover, the absence of the duplication in an unaffected subject born from a female carrier strengthens the significance of the duplication. Little is known on the centromeric 11p15 imprinting region

apart from the ICR2 imprinting control region. One deletion within the L65P duplicated region has been registered as a rare variation (<http://projects.tcag.ca/variation/>; variation 29897) but the parental origin of this deletion is not known. Nevertheless, the 50 kb *cis*-duplication region displays evidence of open chromatin and encompasses two CpG islands and three CTCF binding sites. Analysis of the DNA methylation status of the two CpG islands had not been previously documented. Those two CpG islands are not allele-specific differentially methylated in control subjects, with one being predominantly methylated and the other one unmethylated. In the patient displaying the *cis*-duplication, we showed that the pattern of DNA methylation was similar to the pattern of control subjects. The location of the duplication also corresponds to the centromeric breakpoint of a 1.8 Mb *cis*-duplication responsible for a BWS<sup>33</sup> and the Tm87-16 rhabdoid tumor translocation breakpoint within BWSCR1<sup>36, 37</sup>. All together, this data strongly supports the relevance of the *cis*-duplication in the BWS phenotype and suggests that the *cis*-duplication affects the chromatin conformation and impairs the apposition and/or maintenance of DNA methylation at ICR2.

- Precise analysis of the two imprinting control regions (ICR1 and ICR2) led to the identification of new small deletions. Those deletions were only identified in BWS patients with ICR1 gain of methylation. As for previous identified deletions<sup>18</sup>, the deletion breakpoints in those new cases map repetitive sequences within the B repeats of ICR1, suggesting that these repetitive sequences within ICR1 promote the risk of deletion. No ICR1 deletions were identified in SRS patients with ICR1 loss of methylation and this agrees with previous publications which show the absence of phenotype upon paternal transmission of ICR1 deletions<sup>14, 16</sup>. Moreover, we did not identify ICR2 deletions in this large series of BWS patients with ICR2 loss of methylation. Although ICR2 deletions (0.25 and 0.9 Mb) have been occasionally reported in BWS with loss of methylation at ICR2<sup>19, 20</sup>, the data presented here establishes that they are extremely rare.

- A major advantage of the SNP array is the possibility to combine detection of gain/loss CNVs with copy-neutral CNVs (runs of homozygosity). Such segments of homozygosity could represent

uniparental isodisomy or autozygosity. Uniparental isodisomy is a classical cause of BWS and accounts for 20 to 25% of BWS cases <sup>5 9, 10</sup>, whereas maternal isodisomy has been reported in only one SRS case <sup>11</sup>. When the frequency of uniparental isodisomy is well established in BWS, its extent on chromosome 11 has not been extensively analyzed. In a recent study, Cooper et al. showed that the extent of isodisomy along chromosome 11 is variable but always involves the two 11p15.5 imprinted domains <sup>10</sup>. In this study, we investigated for the first time whether smaller uniparental isodisomies, confined to one of the two 11p15 imprinted domains, might account for DNA methylation defects restricted to ICR1 or ICR2. Although we identified runs of homozygosity in BWS and SRS patients, the B allele frequency was not indicative of mosaicism and there was no mendelian inconsistency when parental DNA was available. This data shows that segmental uniparental isodisomies confined to the *IGF2/H19* or the *KCNQ1OT1/CDKN1C* domains do not account for DNA methylation defects restricted to one of the two 11p15 imprinted domains.

In summary, investigation by SNP array of a large series of BWS and SRS patients with isolated ICR1 or ICR2 DNA methylation defects led to the identification of new molecular defects including novel segmental *cis*-duplications in both SRS and BWS patients and new ICR1 deletions in BWS patients. This study also establishes, for the first time, that segmental uniparental isodisomies do not account for isolated ICR1 or ICR2 DNA methylation defects. We also demonstrate in a large series of BWS patients that ICR2 deletions are not a common mechanism for loss of methylation at ICR2.

Our data also illustrates the difficulty of genetic counseling in patients with 11p15 related growth disorders. Indeed, in more than 50% of cases, the clinical presentation was sporadic rather than familial despite a maternally-inherited genetic defects. This study and a previous one <sup>18</sup> also emphasize that molecular defects in *cis* are more common than initially thought in BWS patients with ICR1 gain of methylation and affect at least 20% of cases.

#### **Supplemental Data:**

Supplemental Data include one figure and two tables and can be found

online at <http://www>.

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**Conflict of interest statement.** None declared.

### **Web Resources:**

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>; International HapMap Project: <http://www.hapmap.org/>; Database of genomic variants: <http://projects.tcag.ca/variation/>; UCSC Genome Browser, March 2006 build, <http://genome.cse.ucsc.edu/>

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**Figures titles and legends:****Figure 1: ICR1 duplication resulting from recombination of a paternal pericentric inversion in a BWS patient with ICR1 gain of methylation**

(A) Graphical overview of B allele frequency and log R Ratio obtained from the SNP array in H47P and his parents, showing a duplication of the *IGF2/H19* domain in H47P. The location of the duplication is highlighted by the grey box. (B) Genomic real-time PCR quantification assay at various loci along the *IGF2/H19* domain validating the duplication. (C) Sequencing at various informative SNPs within the duplication showing that the duplication involves the paternally-inherited allele. (D) Graphical overview of the results obtained from the array-CGH analysis of H47P, showing a distal 11p duplication and a distal 11q deletion. (E) FISH analysis with subtelomeric probes for 11p (green) and 11q (red). The normal chromosome 11 has one green and one red signal for 11p and 11q, respectively. In H47P, the recombinant chromosome 11 [rec(11) dup p inv(11)] has green signals for 11p on both ends and no red signal. In H47P's father, the inverted chromosome 11 [inv(11) p15.5q25] has a red signal on 11p and a green signal on 11q. Small white arrows indicate the centromeres.

**Figure 2: Familial ICR1 duplication in a SRS patient with ICR1 loss of methylation**

(A) Pedigree of the S72P SRS case. (B) Graphical overview of B allele frequency and log R Ratio obtained from the SNP array in S72P and her parents, showing a maternally-inherited segmental duplication of part of the *IGF2/H19* domain in S72P. The location of the duplication is highlighted by the grey box. (C) Genomic real-time PCR quantification assay at various loci along the *IGF2/H19* domain validating the duplication in S72P and her mother. (D) Graphical overview of the results obtained from the array-CGH analysis of S72P. The duplication interval (from 1,530,602 to 2,092,578 bp) is underlined with the pink rectangle. (E) Partial metaphases of patient S72P. FISH with the BAC probe RP11-295K3 within the duplication shows an increased hybridization signal (green signal) on one of the chromosomes 11 (arrow) when the signal with the BAC probe RP3-416J11 outside the duplication (red signal) is similar

on both chromosomes. **(F)** FISH on S72P's interphased nuclei with the BAC probe RP11-295K3 shows three hybridization signals confirming the duplication. **(G)** Chromatograms of the ICR1 B6 repeat including SNP rs61383602 (framed in red) before and after bisulfite treatment for patient S72P and her mother. After bisulfite treatment, patient S72P (non informative for the polymorphism) displays a T allele peak (unmethylated duplicated maternal allele) higher than the C allele peak (methylated paternal allele). In the mother, informative for the polymorphism, before treatment by bisulfite, the C allele peak is higher than the T allele confirming the duplication. The profile is the same after bisulfite treatment showing that the C methylated allele was inherited from the maternal grandfather.

### **Figure 3: Familial ICR2 BWS case**

**(A)** Pedigree of the familial BWS case and segregation. Subjects identified with the duplication are indicated with a star. SB: stillbirth. **(B)** DNA methylation at ICR2, assayed by methyl-sensitive Southern blotting using genomic DNA from the indicated individuals. The upper band (6 kb) is methylated and corresponds to the maternal allele. The lower band (4.2 kb) is unmethylated and corresponds to the paternal (ICR2) allele. **(C)** Graphical overview of log R Ratio results obtained from the SNP array in L65P (III.7), his mother (II.3) and maternal grandmother (I.1), showing a maternally-inherited duplication in L65P and his mother. The location of the duplication is highlighted by the grey box. **(D)** Genomic real-time PCR quantification assay at several loci along the ICR2 domain validating the duplication. **(E)** DNA methylation profiles of the two CpG islands included in the *cis*-duplication determined by bisulfite sequencing in a control subject, the propositus (L65P/III.7), his mother (II.3) and maternal grandmother (I.1). Each line corresponds to an individual cloned DNA fragment and each circle represents a CpG dinucleotide. Methylated CpGs are indicated by filled circles and unmethylated CpGs by open circles. Parental alleles were distinguished by the rs179436 nucleotide polymorphism for the 2,510,680-2,510,907 CpG island and the rs2074247 nucleotide polymorphism for the 2,511,982-2,512,194 CpG island. The 16 and 17 CpG islands are framed.

**Figure 4: ICR1 deletions in BWS patients with ICR1 gain of methylation**

(A) Graphical overview of log R ratio results focused at ICR1 obtained from the SNP array in BWS cases H50P and H63P; location of deletions identified by log R ratio values are underlined with a black arrow. (B) Pedigree of the familial H63P BWS case and segregation. Subjects identified in grey developed Wilms tumors. (C) Agarose gel of PCR amplifications (position 1,976,966 to 1,981,302 bp) showing maternally-inherited deletions in H50P and H63P families. (D) Genomic real-time PCR quantification assay at two loci within the ICR1 domain confirmed the deletions in patients H50P and H63P. (E) Position of the breakpoints of H63P's deletion (at 1,978,248 -1,978,293 bp and 1,980,488-1,980,533 bp) have been identified after sequencing both alleles and are highlighted on the chromatograms. The deletion removes three CTCF binding sites (2, 3 and 4). (F) Localization of ICR1 deletions identified in this study and deletions previously described <sup>15-17</sup>.

**Figure 5:** Runs of homozygosity identified in control subjects and patients. Vertical lines represent length of homozygous regions. The black, light blue, dark blue and red colors represent control subjects, BWS patients with ICR1 gain of methylation, BWS patients with ICR2 loss of methylation and SRS patients with a loss of methylation, respectively. The A light grey box underlines the region encompassing the paternally-imprinted *ASCL2* gene. The B dark grey box underlines the region encompassing the *C11ORF21* and *TSPAN32* genes.

**Supplementary figure 1:** Schematic diagram of the 11p15 region with a summary of the duplications identified in the cohort and duplications previously described <sup>12, 33, 34, 38</sup>.

Table 1: Clinical and molecular features of patients with Beckwith-Wiedemann syndrome

|   | BWS with ICR2          | BWS with ICR1           |
|---|------------------------|-------------------------|
|   | loss of methylation    | gain of methylation     |
| n   | 78                     | 35                      |
| sex F/M   | 36/42                  | 17/18                   |
| gestational age <sup>a</sup> , weeks mean $\pm$ SD; (range) | 36.2 $\pm$ 3.2 (27-41) | 36.7 $\pm$ 3.1 (30-40)  |
| ART, n  | 9                      | 1                       |
| monozygotic twins   | 7                      | 0                       |
| fetal cases   | 6                      | 3                       |
| familial cases  | 1                      | 4 <sup>c</sup>          |
| <b>phenotype</b>  |                        |                         |
| macrosomia at birth <sup>b</sup> , n (%)                    | 31/65 (48%)            | 24/35 (69%)             |
| birth weight (SDS)  | 1.7 $\pm$ 1.6          | 2.8 $\pm$ 1.9           |
| macroglossia, n (%)   | 75/77 (97.5%)          | 31/33 (94%)             |
| abdominal wall defect, n (%)                                | 54/77 (70%)            | 20/32 (62%)             |
| diastasis recti, n (%)                                      | 4                      | 12                      |
| umbilical hernia, n (%)                                     | 21                     | 7                       |
| exomphalos, n (%)   | 29                     | 1                       |
| organomegaly, n (%)   | 33/69 (48%)            | 24/32 (75%)             |
| body asymmetry, n (%)                                       | 20/71 (28%)            | 12/33 (36%)             |
| hypoglycaemia, n (%)  | 26/63 (41%)            | 9/30 (30%)              |
| ear abnormalities, n (%)                                    | 54/74 (73%)            | 9/30 (30%)              |
| tumour, n (%)   | 0/71 (0%)              | 8/32 (25%) <sup>d</sup> |
| <b>others</b>   |                        |                         |
| ICR1 MI, mean $\pm$ (SD)                                    | 52.8 $\pm$ 4.1         | 76 $\pm$ 10.3           |
| (N=53.9 $\pm$ 2%)   |                        |                         |
| ICR2 MI, mean $\pm$ (SD)                                    | 8.5 $\pm$ 7.7          | 47.9 $\pm$ 3.8          |
| (N=51.6 $\pm$ 2.5%)   |                        |                         |
| parental DNA, n(%)  | 62/78 (79.5%)          | 18/35 (51%)             |
| Both parents  | 57                     | 14                      |
| One parent  | 5                      | 4                       |

a: gestational age is in weeks of amenorrhea

b: twins not included

c: 4 subjects from 2 families (two siblings in one family and a son and his mother in the other family)

d: all tumors were Wilms tumors

ART: assisted reproductive technology; MI: methylation index

Table 2: Clinical and molecular features of patients with Silver-Russell syndrome

| <b>SRS with ICR1</b>                  |                            |
|---------------------------------------|----------------------------|
|                                       | <b>loss of methylation</b> |
| n                                     | 72                         |
| sex F/M                               | 25/47                      |
| gestational age <sup>a</sup> , weeks  | 38.2 ± 2                   |
| ART, n                                | 6                          |
| monozygotic twins                     | 3                          |
| <b>phenotype</b>                      |                            |
| IUGR, n (%)                           | 66/68 (97%)                |
| birth weight (SDS) <sup>b</sup>       | -3.1 ± 0.7                 |
| post-natal growth retardation, n (%)  | 61/64 (95%)                |
| relative macrocephaly at birth, n (%) | 59/62 (95%)                |
| facial dysmorphism, n (%)             | 49/57 (86%)                |
| body asymmetry, n (%)                 | 51/68 (75%)                |
| feeding difficulties, n (%)           | 47/63 (75%)                |
| severe, n                             | 16                         |
| moderate, n                           | 31                         |
| BMI (SDS)                             | -2.2 ± 1.1                 |
| BMI ≤ -2 SDS                          | 40/59 (69%)                |
| developmental delay, n (%)            | 9/57 (16%)                 |
| clinodactyly, n (%)                   | 51/61 (84%)                |
| <b>others</b>                         |                            |
| ICR1 MI, mean ± (SD)                  | 23 ± 8.3                   |
| (N=53.9 ± 2%)                         |                            |
| ICR2 MI, mean ± (SD)                  | 47.6 ± 4.1                 |
| (N=51.6 ± 2.5%)                       |                            |
| parental DNA, n(%)                    | 30 (39%)                   |
| both parents                          | 22                         |
| one parent                            | 8                          |

a: gestational age is in weeks of amenorrhea

b: twins not included

ART: assisted reproductive technology; IUGR: intrauterine growth retardation;  
MI: methylation index

Table 3: Copy number variations detected in SRS and BWS patients with cnvPartition v2.4.4 and QuantiSNP v2.3 softwares

| patients | patients' group | CNV type         | origin         | parental chromosome | size kb | markers n | detection cnvPartition/<br>confidence score | detection QuantiSNP /<br>Log Bayes Factor | qPCR validation |
|----------|-----------------|------------------|----------------|---------------------|---------|-----------|---|---|-----------------|
| H4P      | BWS ICR1        | n<br>duplication | NA*            | /                   | 22      | 15        | yes/45.74                                   | no  | no              |
| H47P     | BWS ICR1        | n<br>duplication | <i>de novo</i> | paternal            | 400**   | 526       | yes/1900.99                                 | yes/30.33                                 | yes             |
| L65P     | BWS ICR2        | n<br>duplication | inherited      | maternal            | 50      | 78        | yes/232.11                                  | yes/63.94                                 | yes             |
| L263P    | BWS ICR2        | n<br>deletion    | <i>de novo</i> | maternal            | 4.4     | 3         | yes/73.93                                   | no  | no              |
| S63P     | SRS ICR1        | deletion         | <i>de novo</i> | paternal            | 4       | 3         | yes/230.73                                  | no  | no              |
| S72P     | SRS ICR1        | n<br>duplication | inherited      | maternal            | 80**    | 89        | yes/398.29                                  | yes/44.24                                 | yes             |

\* NA: parental DNA not available

\*\* These CNVs include the most telomeric probe of the OPA.

BWS ICR1: BWS patients with ICR1 gain of methylation. BWS ICR2: BWS patients with ICR2 loss of methylation. SRS ICR1: SRS patients with ICR1 loss of methylation.



Table 4: Characterization of ICR1 deletions in BWS patients with ICR1 gain of methylation

|      | familial history | size (bp) | breakpoints                      | fused repeats | deleted CTCF binding sites |
|------|------------------|-----------|----------------------------------|---------------|----------------------------|
| H32P | no               | 1834      | 1978828-1978846/1980662-1980680  | B6/B3         | 2, 3                       |
| H50P | no               | 1834      | 1978576-1978605 /1980411-1980431 | B6/B3         | 3, 4                       |
| H63P | yes              | 2240      | 1978248 -1978293/1980488-1980533 | B6/B2         | 2, 3, 4                    |

\* Genomic positions were assigned according to the data provided by the UCSC genome browser (hg18, NCBI 36)

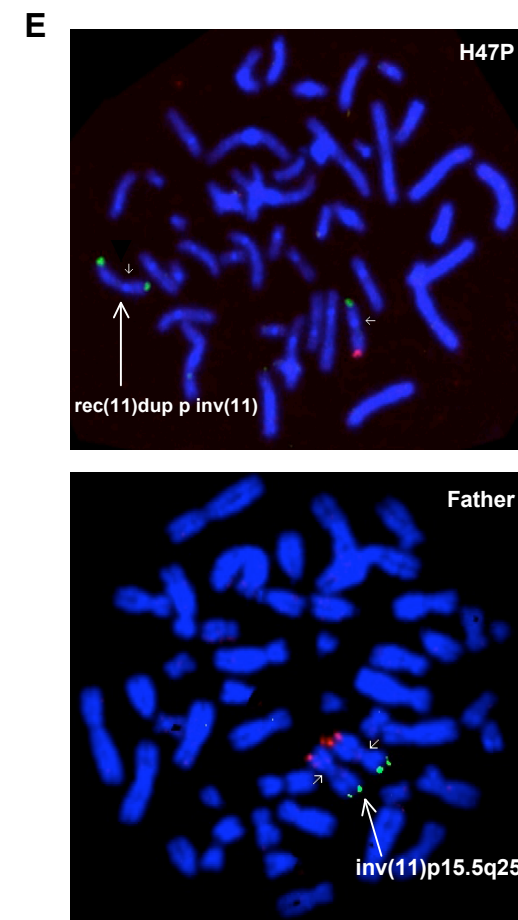
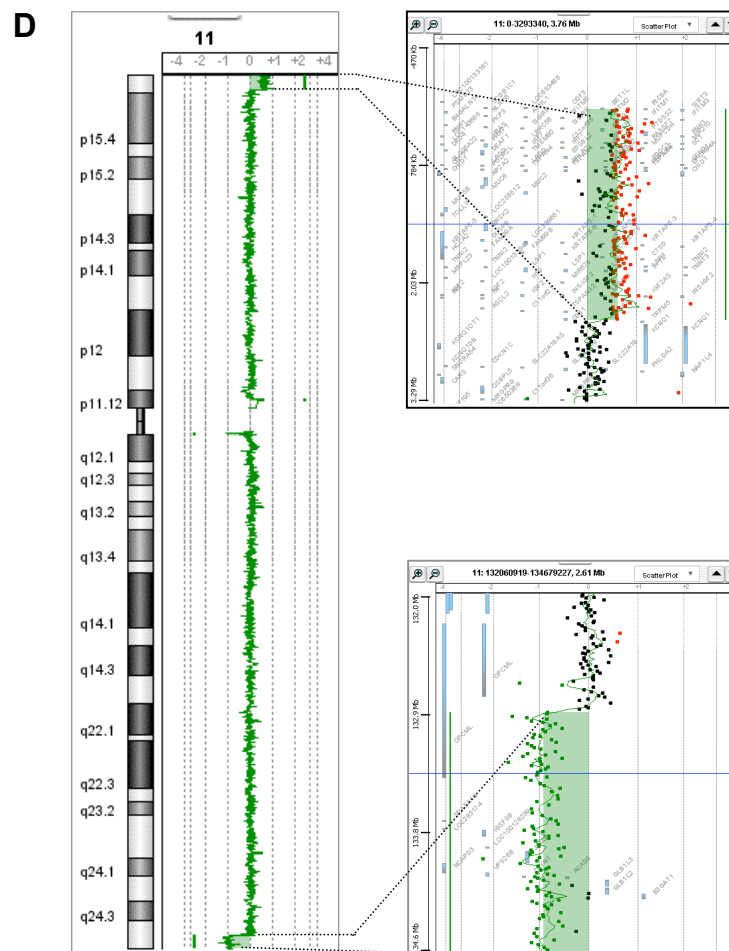
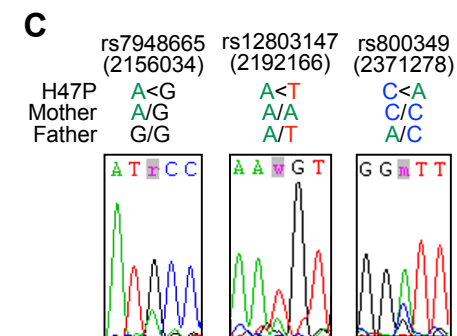
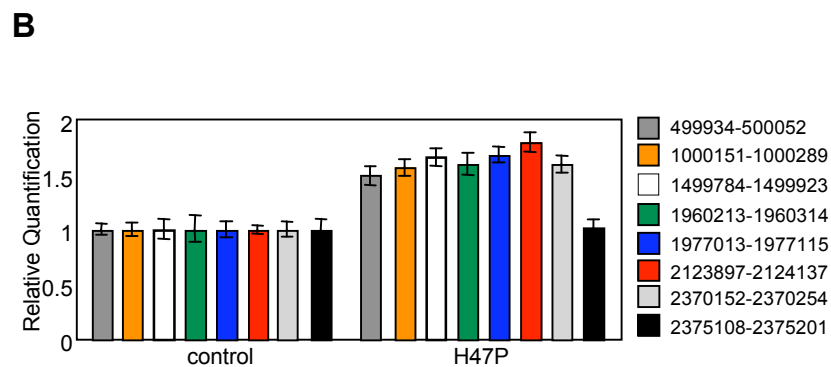
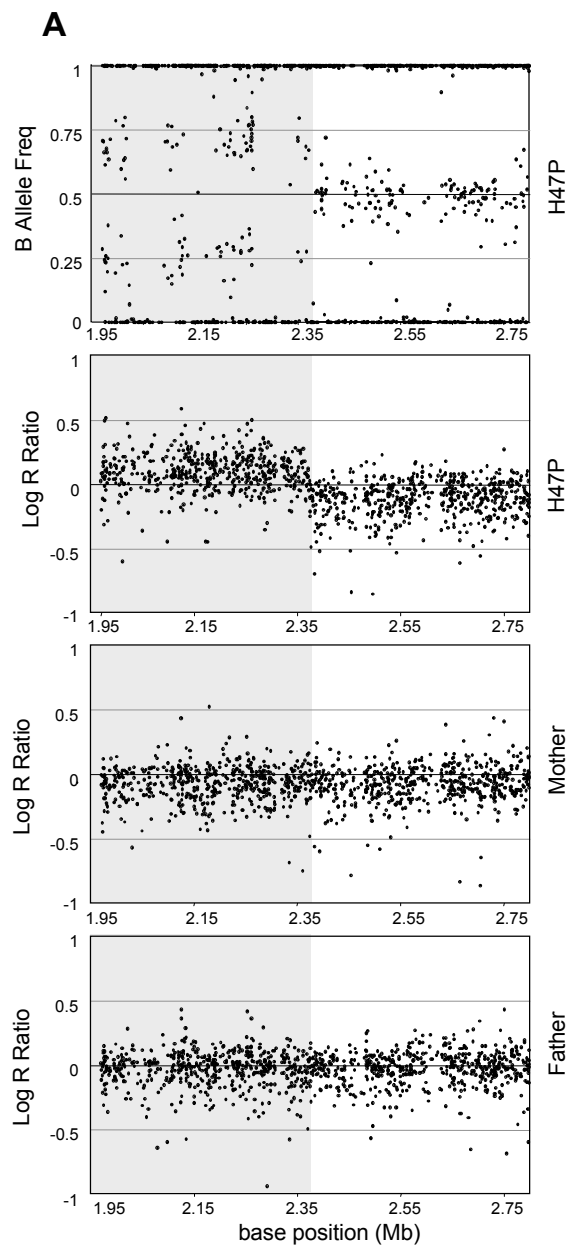
Supplementary table 1: Additional primers used in this study

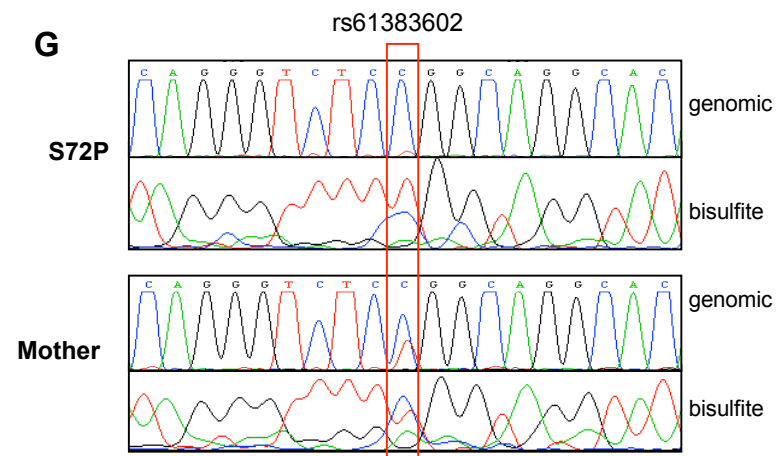
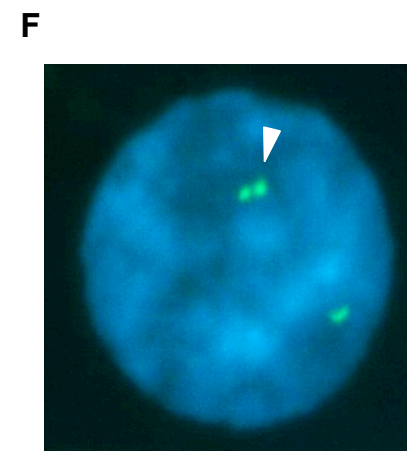
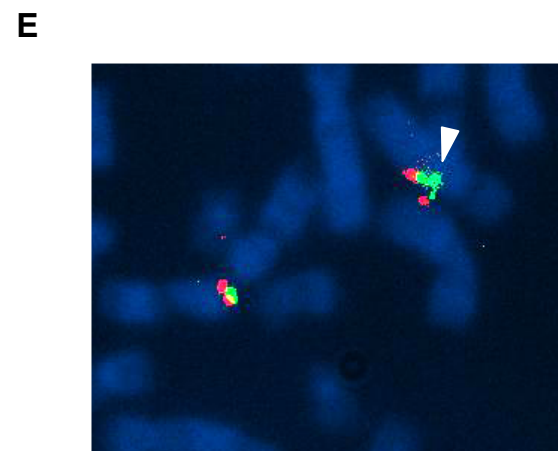
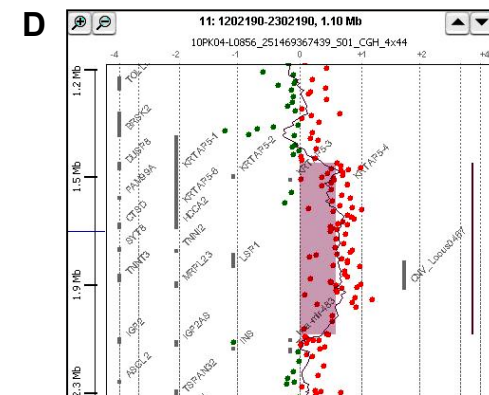
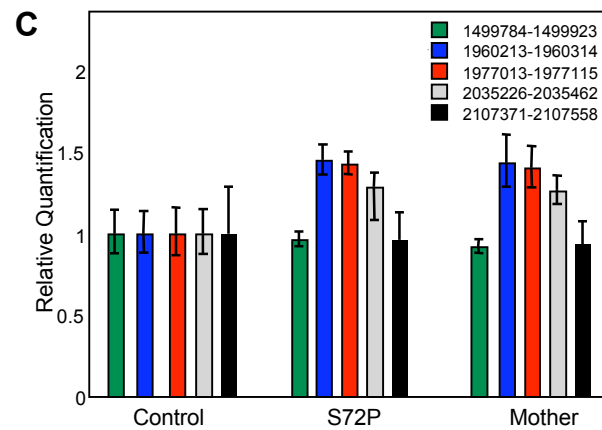
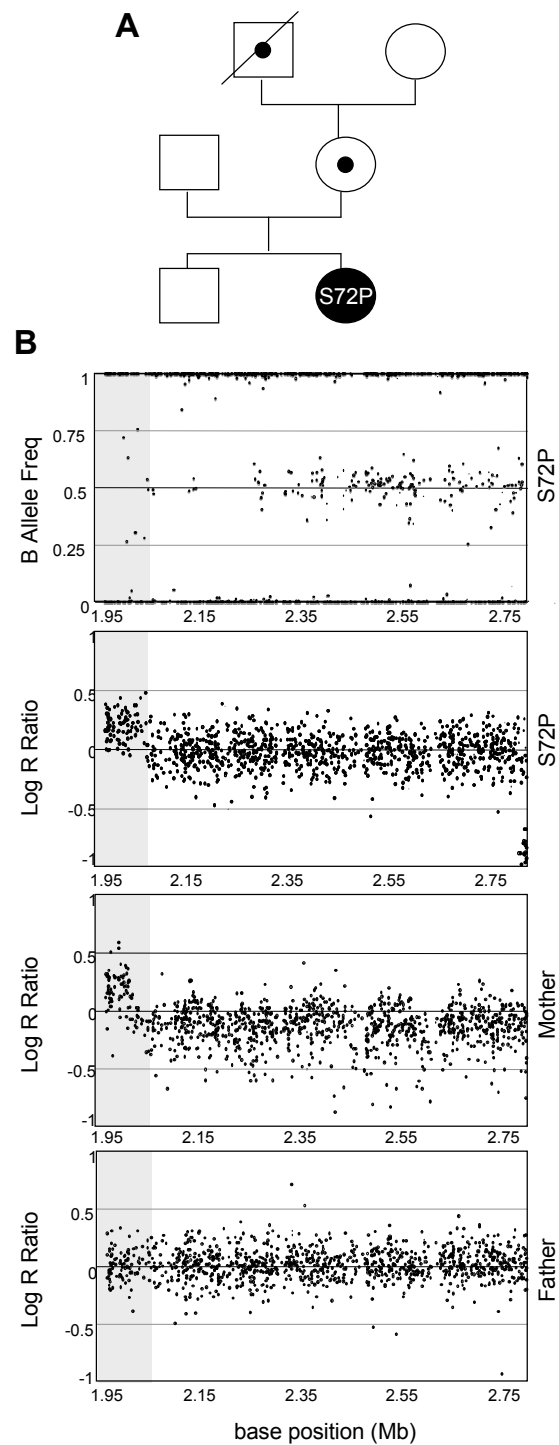
| Name                          | Localization hg18 (bp) | Sequence forward          | Sequence reverse          | Use                                      |
|-------------------------------|------------------------|---------------------------|---------------------------|--|
| hg18_chr11_499934/500052      | 499934-500052          | GAAGTATTCCCACCCCCAGT      | CAGATCTGTGTGGAGCAGGA      | CNV validation H47P, qPCR                |
| hg18_chr11_1000151/1000289    | 1000151-1000289        | TCCCAGCTGTTCTCTCTGT       | TGCAGCACTAACCACACACA      | CNV validation H47P, qPCR                |
| hg18_chr11_1499784/1499923    | 1499784-1499923        | TCTAAGGCTTGGGGTTTTCC      | ACAGCTCGTCCCGTAGAAGA      | CNV validation H47P and S72P, qPCR       |
| hg18_chr11_1960213/1960314    | 1960213-1960314        | GAGGCAAGATTTGGAACGA       | CTGGGCAGGAGACTCTGATT      | CNV validation H47P and S72P, qPCR       |
| hg18_chr11_1977096/1977030    | 1977096-1977030        | ACGTGGAGAGGTGAATTTGC      | TCCTGCTCCAGGCATTGT        | CNV validation H47P and S72P, qPCR       |
| hg18_chr11_2123897/2124137    | 2123897-2124137        | AAACCCACATTCCACAGAGG      | TCTGAGGACCCTTGGAGAAA      | CNV validation H47P, qPCR                |
| hg18_chr11_2370152/2370254    | 2370152-2370254        | GGCAGAAGCAGAGGTCACA       | ACACCATGCAGGTCAGACAC      | CNV validation H47P, qPCR                |
| hg18_chr11_2375108/2375201    | 2375108-2375201        | CCTTCATGCACCTGTCCTTT      | GCATGCCTGATGTTCTTCT       | CNV validation H47P, qPCR                |
| hg18_chr11_2155877/2156345    | 2155877-2156345        | AGGGAAGGGGATCCAGTG        | CAGACACAGACCAGCGAAAA      | CNV validation H47P, Parental origin     |
| hg18_chr11_2371018/2371497    | 2371018-2371497        | CTGCTGAGGGATAGGGGAGT      | AGTGGCATCAGAAGGCACAG      | CNV validation H47P, Parental origin     |
| hg18_chr11_2191887/2192349    | 2191887-2192349        | GTTCTCCTGCCTCCCTAAC       | AGAGCGGGGAAGAAACAGAT      | CNV validation H47P, Parental origin     |
| hg18_chr11_2035226/2035462    | 2035226-2035462        | CCTCGATAGCCATTTTGCAT      | GCATGGCAAAAGGAGAAAAAG     | CNV validation S72P, qPCR                |
| hg18_chr11_2107371/2107558    | 2107371-2107558        | GCTTCTGGGTCTGCTGAC        | CTCGAAGCGTTTTGGATCTC      | CNV validation S72P, qPCR                |
| hg18_chr11_1980297/1980616    | 1980297-1980616        | AGGTGTTTTAGTTTTTGGATGATA  | CCATAAATATTCTATCCCTCACTA  | CNV validation S72P, Bisulfite treatment |
| hg18_chr11_2486398/2486518    | 2486398-2486518        | AGGACCCAGACTCCCAGAC       | AGGTGGTCACTGCCATCTTC      | CNV validation L65P, qPCR                |
| hg18_chr11_2540424/2540567    | 2540424-2540567        | CTGTGTCCACCCCTCTTCAG      | ACTTAGGCCTGGGGACCTT       | CNV validation L65P, qPCR                |
| hg18_chr11_2526198/2526347    | 2526198-2526347        | TCGGTCAGGTTAGTTGCTGT      | CCCCCAGAGCTTAGGACACT      | CNV validation L65P, qPCR                |
| hg18_chr11_2472491/2472581    | 2472491-2472581        | GAAGCCCACTTGATCACCAT      | GAGCACCGATGCAAAAATCT      | CNV validation L65P, qPCR                |
| hg18_chr11_2676672/2677949    | 2676672-2677949        | TGGGACCCCACTACTCAGA       | AGGACACGGCACATCACTTT      | CNV validation L65P, sequencing          |
| hg18_chr11_2677793/2679793    | 2677793-2679793        | ACCGTTCTGCCTGGAGACTG      | GCCACCCTCACTCAACATT       | CNV validation L65P, sequencing          |
| hg18_chr11_2510633/2510968    | 2510633-2510968        | GGTTTTTGATTTTAGTAGAGGGAGG | AAACAAAAACTCCCTAAAAACACAT | CNV validation L65P, Bisulfite treatment |
| hg18_chr11_2511951/2512483    | 2511951-2512483        | AGTGAGATTTTGTAGGGAGTTTTTG | AACCCACAAAAAACACAACCTCT   | CNV validation L65P, Bisulfite treatment |
| hg18_chr11_2674923/2675237    | 2674923-2675237        | GGAGTAGGCCAAGGATGTCA      | GAGTTTTCCAGAGGCAGCAC      | deletion ICR2 L65P, qPCR                 |
| hg18_chr11_2649043/2649262    | 2649043-2649262        | GACCTCAAAATCCGATGTCC      | GGTTGCTCTTCTGCCTGCTA      | deletion ICR2 L65P, qPCR                 |
| hg18_chr11_1978454/1978696    | 1978454-1978696        | ATGTGGCTCCCATGAATGTC      | GGCTCTTGCATAGCACATGA      | deletions ICR1 H50P and H63P, qPCR       |
| hg18_chr11_1979289/1979612    | 1979289-1979612        | CTGATTCCAGCAGCACAGAG      | TCAGTGCAGGTTTGAGATGC      | deletions ICR1 H50P and H63P, qPCR       |
| hg18_chr5_134898593/134898695 | 134898593-134898695    | GCTCTCTGACCCCAAGTAGCC     | TGTGTGGAGCAAGTCTTTGG      | housekeeping gene, qPCR                  |

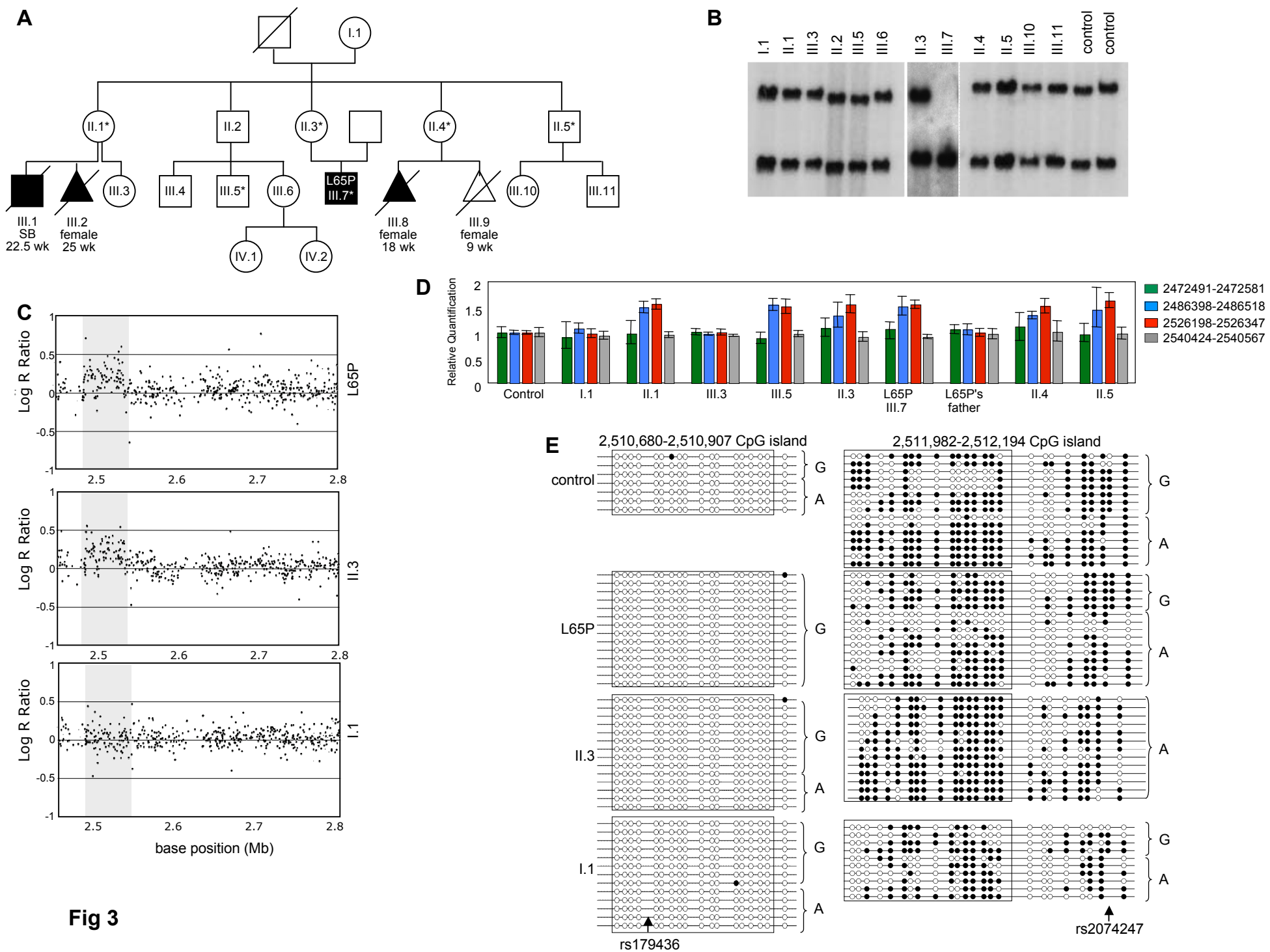
Supplementary table 2: Runs of homozygosity detected in control subjects and patients

| Sample id.             | Start Position (bp) | End Position (bp) | Length (bp) | Probes (n) | Score   | Max. Log BF |
|------------------------|---------------------|-------------------|-------------|------------|---------|-------------|
| <u>Control samples</u> |                     |                   |             |            |         |             |
| CS_107                 | 2264880             | 2381611           | 116732      | 210        | 37.1696 |             |
| CS_154                 | 2155554             | 2261555           | 106002      | 195        | 32.2554 |             |
| CS_187                 | 2559602             | 2690701           | 131100      | 195        | 34.3885 |             |
| CS_189                 | 2283188             | 2381611           | 98424       | 168        | 30.3942 |             |
| CS_19                  | 2128498             | 2346891           | 218394      | 408        | 101.166 |             |
| CS_205                 | 2128498             | 2261555           | 133058      | 257        | 45.1774 |             |
| CS_245                 | 2159621             | 2261078           | 101458      | 185        | 35.3047 |             |
| CS_254                 | 2155554             | 2262744           | 107191      | 199        | 33.8643 |             |
| CS_300                 | 2155554             | 2347625           | 192072      | 349        | 62.0078 |             |
| CS_300                 | 2780921             | 2869522           | 88602       | 176        | 42.9269 |             |
| CS_315                 | 2233543             | 2381611           | 148069      | 278        | 37.1292 |             |
| CS_365                 | 2233543             | 2529468           | 295926      | 507        | 62.7432 |             |
| CS_370                 | 2707910             | 2816117           | 108208      | 207        | 38.2093 |             |
| CS_375                 | 2264880             | 2381611           | 116732      | 210        | 40.529  |             |
| CS_415                 | 2412354             | 2537338           | 124985      | 188        | 37.1774 |             |
| CS_7                   | 2156905             | 2267446           | 110542      | 205        | 30.2087 |             |
| CS_D                   | 2165989             | 2381611           | 215623      | 394        | 59.2346 |             |
| <u>ICR1 GOM BWS</u>    |                     |                   |             |            |         |             |
| AA30P                  | 2283727             | 2479634           | 195908      | 302        | 32.303  |             |
| H12P                   | 2286571             | 2381611           | 95041       | 158        | 30.3052 |             |
| H20P                   | 2285283             | 2381284           | 96002       | 161        | 34.5552 |             |
| H23P                   | 2283727             | 2425532           | 141806      | 239        | 33.3802 |             |
| H29P                   | 2176538             | 2387096           | 210559      | 381        | 72.3474 |             |
| H2P                    | 2151386             | 2261555           | 110170      | 203        | 42.128  |             |
| H2P                    | 2291307             | 2387096           | 95790       | 159        | 39.9038 |             |
| H43P                   | 1970084             | 2177516           | 207433      | 358        | 78.7391 |             |
| H43P                   | 2273296             | 2389540           | 116245      | 209        | 49.8905 |             |
| H43P                   | 2409470             | 2597296           | 187827      | 290        | 61.554  |             |
| H44P                   | 2156905             | 2381611           | 224707      | 407        | 90.1299 |             |
| H54P                   | 2181257             | 2267728           | 86472       | 156        | 40.1736 |             |
| H55P                   | 2206725             | 2381611           | 174887      | 312        | 43.1212 |             |
| H61P                   | 2283727             | 2385251           | 101525      | 172        | 31.1806 |             |
| H61P                   | 2644661             | 2782775           | 138115      | 264        | 53.5701 |             |
| H69P                   | 2511521             | 2708236           | 196716      | 304        | 40.6492 |             |
| H6P                    | 2047032             | 2381611           | 334580      | 592        | 152.827 |             |
| H70P                   | 2505433             | 2690701           | 185269      | 289        | 39.761  |             |
| H8P                    | 2144814             | 2261555           | 116742      | 217        | 49.5353 |             |
| <u>ICR2 LOM BWS</u>    |                     |                   |             |            |         |             |
| 41P                    | 2138346             | 2381611           | 243266      | 449        | 91.8929 |             |
| 41P                    | 2423780             | 2597296           | 173517      | 269        | 31.3046 |             |
| L101P                  | 2156905             | 2381611           | 224707      | 406        | 88.2207 |             |
| L110P                  | 2073262             | 2261555           | 188294      | 344        | 78.5962 |             |
| L113P                  | 2191119             | 2387441           | 196323      | 342        | 71.585  |             |
| L116P                  | 2206725             | 2381284           | 174560      | 312        | 45.4417 |             |
| L119P                  | 2497741             | 2720353           | 222613      | 354        | 65.004  |             |
| L134P                  | 2582824             | 2751568           | 168745      | 270        | 34.6159 |             |
| L138P                  | 2716781             | 2869522           | 152742      | 295        | 52.1911 |             |
| L151P                  | 2173007             | 2262744           | 89738       | 167        | 31.3149 |             |
| L164P                  | 2165989             | 2381284           | 215296      | 393        | 75.3495 |             |
| L167P                  | 2399757             | 2584063           | 184307      | 290        | 54.5854 |             |
| L170P                  | 2150393             | 2272679           | 122287      | 226        | 50.2314 |             |
| L173P                  | 2285283             | 2388145           | 102863      | 175        | 35.2167 |             |
| L177P                  | 2283188             | 2387096           | 103909      | 179        | 35.2186 |             |
| L177P                  | 2559602             | 2720353           | 160752      | 248        | 40.4669 |             |
| L186P                  | 2185285             | 2387096           | 201812      | 363        | 60.9426 |             |

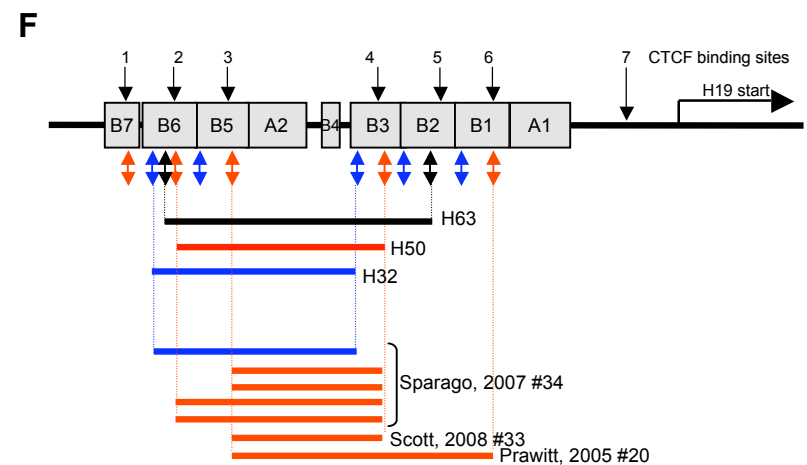
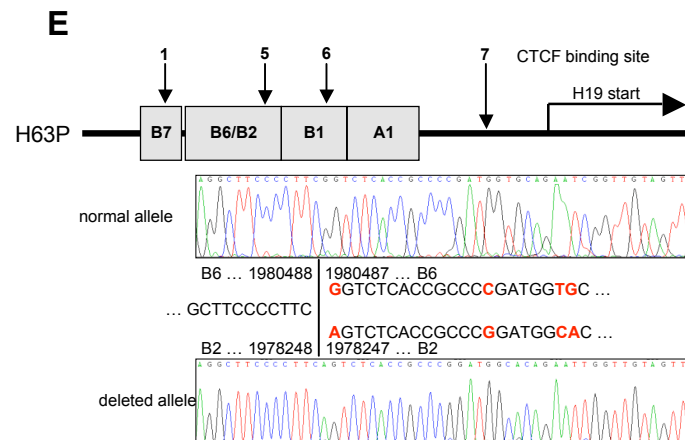
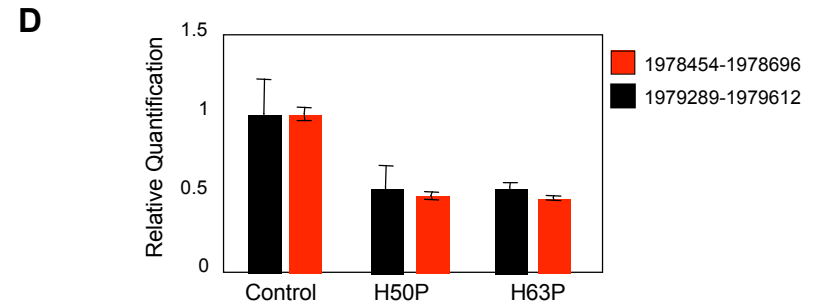
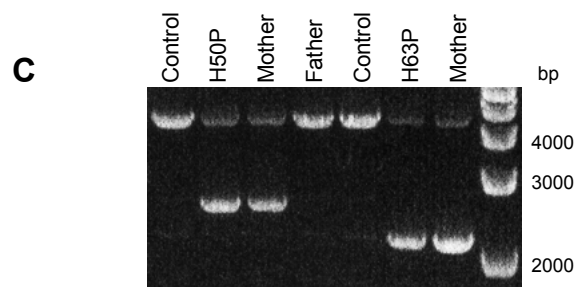
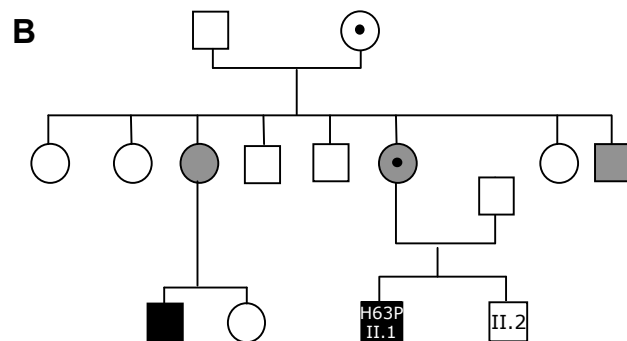
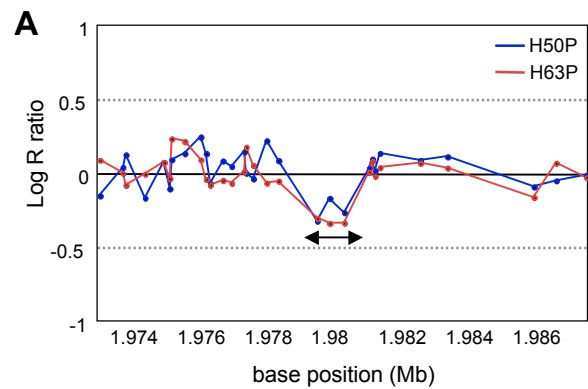
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|--------------|---------|---------|--------|-----|---------|
| L190P        | 2264880 | 2387096 | 122217 | 221 | 44.6633 |
| L196P        | 2264880 | 2387096 | 122217 | 221 | 45.4819 |
| L1P          | 2156905 | 2347625 | 190721 | 346 | 47.3695 |
| L203P        | 2264880 | 2388145 | 123266 | 223 | 41.479  |
| L20P         | 2283188 | 2387096 | 103909 | 179 | 33.5019 |
| L214P        | 2250529 | 2529468 | 278940 | 472 | 101.227 |
| L246P        | 2283727 | 2381611 | 97885  | 166 | 30.5518 |
| L262P        | 2270670 | 2381611 | 110942 | 200 | 35.9644 |
| L278P        | 2250529 | 2381611 | 131083 | 244 | 49.4586 |
| L39P         | 2126011 | 2261555 | 135545 | 266 | 38.9765 |
| L39P         | 2393143 | 2505833 | 112691 | 169 | 33.0644 |
| L42P         | 2168253 | 2381611 | 213359 | 390 | 72.101  |
| L49P         | 2176538 | 2267728 | 91191  | 168 | 30.8099 |
| L4P          | 2000375 | 2191705 | 191331 | 332 | 44.9354 |
| L4P          | 2233543 | 2381611 | 148069 | 278 | 36.2062 |
| L4P          | 2652083 | 2764038 | 111956 | 206 | 39.2673 |
| L58P         | 2176538 | 2288613 | 112076 | 220 | 46.4965 |
| L95P         | 2039703 | 2262744 | 223042 | 386 | 111.035 |
| <hr/>        |         |         |        |     |         |
| ICR1 LOM SRS |         |         |        |     |         |
| S103P        | 2049559 | 2192166 | 142608 | 263 | 63.3619 |
| S41P         | 2060139 | 2177516 | 117378 | 217 | 55.3193 |
| S29P         | 2104733 | 2192830 | 88098  | 203 | 32.0159 |
| S5P          | 2130774 | 2261555 | 130782 | 252 | 48.5926 |
| S122P        | 2133080 | 2241585 | 108506 | 207 | 36.0915 |
| S129P        | 2137348 | 2246000 | 108653 | 205 | 30.2934 |
| S72P         | 2149268 | 2261555 | 112288 | 208 | 42.3326 |
| S116P        | 2155554 | 2261555 | 106002 | 194 | 41.4788 |
| S107P        | 2156905 | 2267446 | 110542 | 205 | 46.4942 |
| S21P         | 2156905 | 2425532 | 268628 | 480 | 118.579 |
| S75P         | 2156905 | 2261555 | 104651 | 192 | 45.9219 |
| S121P        | 2159621 | 2262915 | 103295 | 192 | 44.5157 |
| S115P        | 2165989 | 2292816 | 126828 | 250 | 65.862  |
| S104P        | 2191119 | 2387096 | 195978 | 341 | 74.2499 |
| S120P        | 2191119 | 2308917 | 117799 | 218 | 50.8112 |
| S123P        | 2233543 | 2385251 | 151709 | 284 | 68.3263 |
| S38P         | 2233543 | 2440576 | 207034 | 372 | 67.4105 |
| S95P         | 2233543 | 2387096 | 153554 | 289 | 49.5405 |
| S41P         | 2242435 | 2445918 | 203484 | 358 | 79.6654 |
| S18P         | 2253669 | 2381284 | 127616 | 234 | 42.2847 |
| S127P        | 2264880 | 2405095 | 140216 | 253 | 52.8612 |
| S84P         | 2264880 | 2425532 | 160653 | 281 | 68.3503 |
| S32P         | 2270670 | 2426669 | 156000 | 276 | 36.1267 |
| S20P         | 2276509 | 2385251 | 108743 | 194 | 32.2157 |
| S88P         | 2281815 | 2385251 | 103437 | 178 | 31.6654 |
| S117P        | 2283727 | 2384402 | 100676 | 171 | 38.3117 |
| S60P         | 2283727 | 2387096 | 103370 | 176 | 38.4677 |
| S20P         | 2409470 | 2594837 | 185368 | 286 | 33.6277 |
| S120P        | 2423780 | 2543930 | 120151 | 187 | 39.6514 |



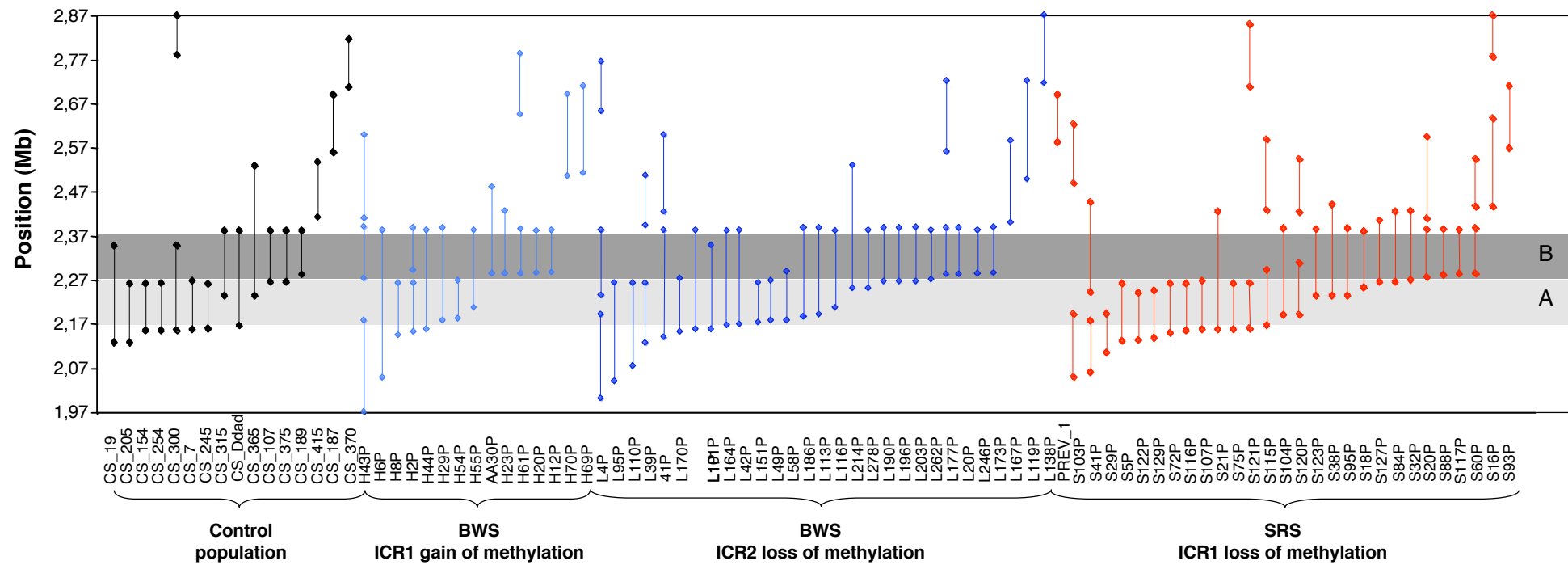


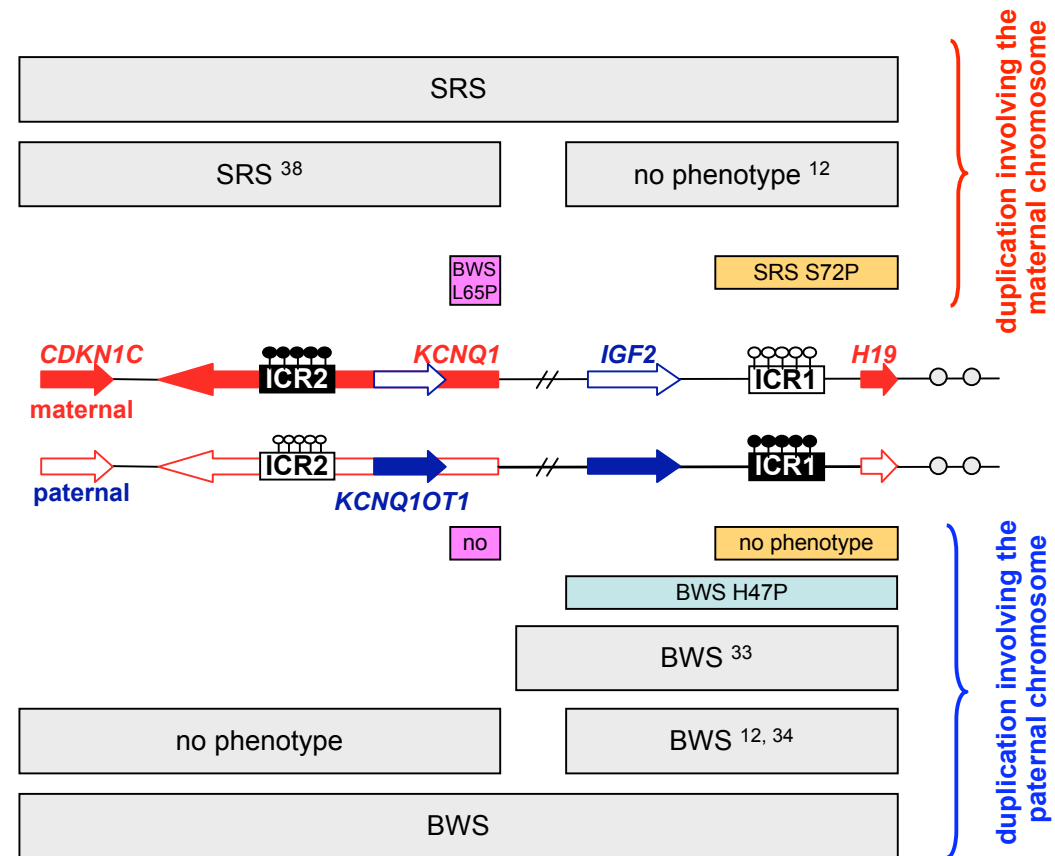


**Fig 3**









Suppl fig 1